ORIGINAL ARTICLE

3-Methylindole production is regulated in *Clostridium scatologenes* ATCC 25775

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Abstract

Aims: 3-Methylindole (3-MI) is a degradation product of L-tryptophan and is both an animal waste malodorant and threat to ruminant health. Culture conditions influencing 3-MI production in *Clostridium scatologenes* ATCC 25775 were investigated.

Methods and Results: Extracellular 3-MI levels in cells cultured in brain heart infusion (BHI) medium (pH 7·0) at 33°C and 37°C for 72 h were 907 ± 38 and 834 ± 121 μmol l^{-1}, respectively. Cells cultured in tryptone-yeast (TY) extract medium at 37°C for 48 h produced 104 ± 86 μmol l^{-1} 3-MI; however, addition of 1 mmol l^{-1} L-tryptophan failed to increase extracellular levels (113 ± 50 μmol l^{-1} 3-MI). Specific activity of indole acetic acid decarboxylase measured in BHI, TY and TY plus 1 mmol l^{-1} tryptophan-grown cells displayed 35-, 33- and 76-fold higher levels than in semi-defined medium-grown cells.

Conclusions: When cultured in rich medium, at 33°C or 37°C and pH 7·0, *Cl. scatologenes* ATCC 25775 optimally produced 3-MI. Addition of L-tryptophan to medium did not lead to significant increases in extracellular 3-MI levels. Whole cell assays indicate growth in rich medium significantly up-regulated 3-MI production.

Significance and Impact of the Study: Information presented here may prove useful in understanding what factors influence 3-MI production in malodorous animal wastes.

Introduction

3-Methylindole (3-MI, skatole) is an endproduct of anaerobic bacterial L-tryptophan catabolism and a problematic component in the food animal industries. 3-MI causes acute pulmonary oedema and emphysema in ruminants, such as cattle and goats (Carlson *et al.* 1972; Yokoyama *et al.* 1975) and contributes to the offensive odours of boar taint that are generated when pork from intact male swine is cooked. 3-MI is one of many compounds responsible for malodours associated with food animal production and waste (Mackie *et al.* 1998). 3-MI is present in the rumen headspace (Cai *et al.* 2006), swine lagoons (Loughrin *et al.* 2006), swine and human faeces (Moore *et al.* 1987; Hawe *et al.* 1993) and exhaust air

from swine facilities (Wright *et al.* 2005) and has a low detection threshold value (Schiffman *et al.* 2001; Greenman *et al.* 2004).

While much work identifies 3-MI as an important factor in animal production, limited progress has been achieved in understanding 3-MI biosynthesis. This is largely because of lack of bacterial strains which produce 3-MI. Rosenberger (1959) isolated five clostridial strains which produce 3-MI and Spray (1948) reported that *Cl. naseum* produces 3-MI when grown in rich medium, however, these strains are not readily available. Yokoyama *et al.* (1977) isolated *Lactobacillus* spp. and Attwood *et al.* (2006) isolated four strains from other bacterial species from the bovine rumen which produced 3-MI from indole acetic acid (IAA), the penultimate compound in

3-MI production. However, only a few strains have been reported to produce 3-MI from tryptophan. Recently, two strains of ruminal bacteria have been isolated which produce 3-MI in the absence of IAA (Attwood *et al.* 2006) in rich medium, however, *Clostridium scatologenes* ATCC 25775 is the best studied bacterial strain known to be capable of 3-MI production from tryptophan (Fellers and Clough 1925; Elsden *et al.* 1976; Elsden and Hilton 1978, 1979; Jensen *et al.* 1995; Kusel *et al.* 2000; Liou *et al.* 2005). It is an objective of this laboratory to elucidate regulatory factors which influence 3-MI production in axenic bacterial cultures as this information could lead to a greater understanding of 3-MI production in animal wastes. The pathway of tryptophan conversion into 3-MI has been elucidated in *Cl. scatologenes* ATCC 25775 (Whitehead *et al.* 2008) and it is this strain we have chosen to further characterize the effects of temperatures, media pH values and media formulation on 3-MI production.

Materials and methods

Clostridium scatologenes ATCC 25775 was obtained from American Type Culture Collection (Manassas, VA, USA). All media were prepared and maintained under strict anaerobic conditions (Holdeman *et al.* 1977) at pH 7·0 and incubated at 37°C, unless otherwise noted. To determine the effects of temperature, pH and medium constituents on cell growth and 3-MI production, cultures were passed for no fewer than three successive transfers in the test conditions prior to inoculation of experimental cultures. Cell growth was monitored at absorbance at 660 nm (A_{660nm}) using a Shimadzu UV mini-1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). To ensure all spectrophotometric data were taken within the linear range of the instrument, samples were diluted, when necessary, to yield values between 0·05 and 1·0 absorbance units.

Media contained (per litre) 1·0 mg resazurin, 2·0 mg haemin, 40 ml salt solution (Holdeman *et al.* 1977) and 1·0 g cysteine-HCl. Additional ingredients (per litre) in brain heart infusion (BHI) were 37 g BHI including glucose (Bacto Becton Dickinson, Franklin Lakes, NJ, USA), 2 g fructose, 10 g yeast extract (Bacto) and 2·69 g sodium bicarbonate. Additional ingredients (per litre) in tryptone-yeast (TY) extract medium (Holdeman *et al.* 1977) were 20 g tryptone (Fisher Scientific, Waltham, MA, USA), 10 g yeast extract and 1 μl Vitamin K_1 (Sigma Chemical Company, St Louis, MO, USA). Additional ingredients (per litre) in semi-defined (SD) (Elsden *et al.*) medium were 10 mmol glucose, 2 g yeast extract, 5 g ammonium sulfate, 2·69 g sodium bicarbonate and 1 ml modified trace mineral solution (McInerney *et al.* 1979;

Genthner *et al.* 1981). Where indicated, 20 g replaced 2 g of yeast extract in SD medium.

BHI medium was used for experiments determining the effects of incubation temperature and pH on growth and 3-MI production. SD medium was also modified by inclusion of 1% peptone (Difco, Detroit, MI, USA) or 1% casamino acids (Difco), each replacing glucose. SD and the stated modifications of SD medium were used to compare growth and 3-MI levels and were conducted at pH 7·0°C and 37°C. L-Tryptophan was suspended in water, filter-sterilized using a 0·2-μm pore-size filter (Fisher Scientific) and added to medium following autoclaving. All chemicals were purchased from a commercial vendor and were of technical grade or higher.

For HPLC determination of 3-MI concentrations, culture samples were subjected to centrifugation (16 000 *g*; 10 min; 4°C) to pellet cells and the supernatant removed. One volume of acetonitrile was added to the supernatant. Culture supernatant and whole cell assay samples were chilled at −20°C for 20 min and then centrifuged (16 000 *g*; 10 min; 4°C). The resulting supernatant was filtered through a 0·45-μm pore-size membrane (Fisher Scientific) and diluted, as appropriate, with acetonitrile to ensure 3-MI levels were within the linear range of detection. Samples were analysed immediately after processing or stored at −20°C in amber-glass vials (National Scientific Company, Rockwood, TN, USA) until analysed. A Waters Corporation (Milford, MA, USA) HPLC was used for detection and quantification of 3-MI. The system included an inline solvent degasser, 1525 gradient pump, 717 plus autosampler and 2475 multi-wavelength fluorescence detector with data collected and analysed using Empower software (Waters Corp.). A Sunfire C-18 reverse phase column (4·6 × 150 mm; Waters Corp.) was equilibrated in 0·1 mol l^{-1} ammonium acetate (pH 6·0) and at 5-min post-injection, a linear gradient to 100% acetonitrile was executed over the course of 10 min. Flow rate was 1·0 ml min^{-1}. Sample injection volume was 0·01 ml. Aqueous solvents were filtered through a 0·2-μm pore-size filter. Both aqueous and organic solvents were degassed by vacuum prior to application. Data were collected at the excitation and emission wavelengths of 285 and 340 nm, respectively. Identification of analytes was achieved by comparing retention times to authentic standards. Analytes were quantified by comparison to a standard curve prepared using authentic standards. The limit of detection of 3-MI was 0·08 pmol with linear regression analysis yielding an *r*-squared value of 0·98. Ratios of 3-MI levels to cell density were calculated by dividing the concentration of 3-MI, expressed in μmol l^{-1}, by the A_{660nm} value.

Suspensions of *Cl. scatologenes* ATCC 25775 whole cells were assayed for 3-MI production using a modification of

a previously published method (Honeyfield and Carlson 1990). Sample preparation and assays were performed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA) maintained with a 5% H_2 and 95% CO_2 atmosphere. Cultures were grown overnight at 37°C, decanted into sterile screw cap polypropylene tubes, subjected to centrifugation (5500 *g*; 15 min; 4°C) outside the chamber and then immediately returned to the chamber. In a similar fashion, the cell pellet was washed three times in chilled 2X Tris-ADS buffer inside the anaerobic chamber, centrifuged (3000 *g*; 10 min; 4°C) and then returned to the chamber. Cells were resuspended to a final concentration of 40 A_{660nm} and diluted in the assay mixture to no less than 8 A_{660nm}. Specific activity values were standardized to 1 ml of a cell suspension of 1·0 A_{660nm}. Assays were conducted in the presence of 1 mmol l^{-1} IAA in 16 mm × 125 mm borosilicate tubes and maintained at 37°C for 1·5–3 h. Reactions were initiated by addition of cells and terminated by addition of an equal volume of chilled acetonitrile and processed for HPLC analysis. Specific rates of IAA were corrected for background levels of 3-MI which were determined by addition of acetonitrile prior to addition of cells.

For GC/MS analysis, culture supernatant was prepared by pelleting cells by centrifugation (16 000 *g*; 10 min; 4°C), placed in screw cap vials (Kimble Glass, Inc) and frozen at −20°C until analysed (Loughrin 2006). Statistical analysis was performed using the Microsoft Office Excel 2003 software package.

Results

GC/MS analysis was initially used to determine extracellular malodorants produced by *Cl. scatologenes* ATCC 25775 grown overnight at 37°C, pH 7·0, in BHI. Data indicate 276 ± 9·9 and 22·3 ± 6·0 μmol l^{-1} of 3-MI and 4-methylphenol, respectively, were present and phenol, 4-ethylphenol and indole were absent. Similar estimations of 3-MI concentrations were achieved using HPLC (Table 1), therefore HPLC was used for routine 3-MI analysis.

Clostridium scatologenes ATCC 25775 was cultured at various temperatures in BHI (pH 7·0) medium while monitoring cell density and 3-MI levels (Fig. 1). Consistent with previous reports (Liou *et al.* 2005), robust growth was observed at temperatures of 33°C, 37°C and 40°C with growth peaking at 24 h of incubation while growth at 24°C peaked at 48 h (Fig. 1a). 3-MI production was observed at all temperatures supporting growth with the highest levels at 907 ± 38 and 834 ± 121 μmol l^{-1} at 33°C and 37°C, respectively, after 72 h (Fig. 1b). Production of 3-MI was greatly diminished by culturing at 24°C.

The effect of medium pH on growth at 37°C and 3-MI production was determined in BHI medium (Fig. 2). Initial medium pH values of 6·0 and 7·0 yielded maximum cell density values of 4·4 ± 0·12 and 3·2 ± 0·08, respectively, at 24 h while pH 5·0 yielded a maximum value of 2·0 ± 0·04 at 72 h (Fig. 2a). Medium adjusted to pH 8·0 failed to support growth. Maximum 3-MI levels (596 ± 62 μmol l^{-1}) were observed at 48 h in pH 7·0 medium (Fig. 2b). While pH 6·0 medium displayed the greatest cell growth, 3-MI levels remained substantially below the pH 7·0 medium at all time points tested.

Growth of *Cl. scatologenes* ATCC 25775 in BHI medium for 72 h produced the highest concentration of 3-MI (328·86 ± 24·14 μmol l^{-1}) of all media tested (Table 1). TY medium produced substantial amounts of 3-MI (113·48 ± 37·19 μmol l^{-1}) and addition of 1 mmol l^{-1} L-tryptophan failed to significantly increase 3-MI levels. When more austere growth conditions were imposed, such as SD, SD + 1% peptone, or SD + 1% casamino acids, little 3-MI was produced although the cultures grew to appreciable densities (Table 1). SD medium alone supported appreciable growth (0·25 A_{660nm}) although only trace amounts of 3-MI were produced. SD medium with 1% casamino acids produced small amounts of 3-MI (3·18 ± 1·4 μmol l^{-1}) although grew to 0·42 A_{660nm} and SD medium with 1% peptone produced 9·93 ± 3·92 μmol l^{-1} 3-MI and grew to 0·39 A_{660nm}. However, increasing yeast extract from 0·2% to 2·0% in SD medium had substantial effects on cell densities (0·25 *vs* 1·1 A_{660nm}) as well as increasing levels of 3-MI from trace amounts to 63·71 ± 21·72 μmol l^{-1} (*P* = 0·002). Ratios of 3-MI levels to cell density varied from 237·7 for TY with 1 mmol l^{-1} L-tryptophan to 0·6 for SD medium (Table 1), representing a 396-fold difference.

3-MI production rates were determined in anaerobic whole cell assays, using 1 mmol l^{-1} IAA as the substrate. IAA decarboxylase, the final step in 3-MI synthesis, is similar to 4-hydroxyphenylacetate decarboxylase, the final step in 4-methyl phenol (p-cresol) synthesis, which is an oxygen-sensitive, glycyl radical enzyme (Selmer and Andrei 2001; Yu *et al.* 2006). Hence, we maintained strict anaerobic conditions by conducting the assay in an anaerobic chamber. Rates of endogenous 3-MI production, as determined by omission of IAA from the reaction mixture, were similar for BHI, TY and TY + 1 mmol l^{-1} tryptophan. Values ranged from 0·007 to 0·011 nmol 3-MI h^{-1} ml of culture at 1·0 A660 nm^{-1}. Endogenous 3-MI production in SD-grown cells was low, as levels were below the linear range for quantification but were often detectable (Table 2). Specific rates of 3-MI production were determined by inclusion of 1 mmol l^{-1} IAA in the reaction mixtures. Cells cultured in TY or TY with 1 mmol l^{-1} L-tryptophan displayed rates of 0·033 ± 0·012

Table 1 Levels of 3-methylindole in culture supernatant of *Clostridium scatologenes* ATCC 25775 grown using various media at 37°C, pH 7·0

Medium (n)	0 h		24 h		48 h		72 h	
	3MI (μmol l^{-1})	A_{660nm}	3MI (μmol l^{-1})	A_{660nm}	3MI (μmol l^{-1})	A_{660nm}	3MI (μmol l^{-1})	A_{660nm}
BHI (3)	0·06 ± 0·00*[0·33]	0·18 ± 0·00	207·6 ± 5·68[67·0]	3·10 ± 0·10	307·6 ± 4·46[130·0]	2·36 ± 0·14	328·86 ± 24·14[199·5]	1·65 ± 0·10
TY (4)	2·75 ± 1·45[23·6]	0·12 ± 0·04	15·41 ± 5·95[23·76]	0·65 ± 0·07	104·86 ± 55·57[135·5]	0·77 ± 0·09	113·48 ± 37·19[147·6]	0·77 ± 0·04
TY + 1 mM Trp† (4)	5·62 ± 2·77[47·1]	0·12 ± 0·05	42·57 ± 23·26[54·1]	0·79 ± 0·09	113·14 ± 49·75[133·2]	0·85 ± 0·06	186·57 ± 42·63[237·7]	0·78 ± 0·04
SD‡ (6)	N.D.§	0·05 ± 0·00	0·06 ± 0·00[0·1]	0·56 ± 0·04	0·08 ± 0·01[0·27]	0·31 ± 0·04	0·14 ± 0·00[0·6]	0·25 ± 0·01
SD + 2·0% YE¶ (3)	0·57 ± .37[4·1]	0·14 ± 0·00	20·35 ± 4·13[13·6]	1·50 ± 0·04	81·57 ± 30·37[67·9]	1·20 ± 0·06	63·71 ± 21·72[57·9]	1·10 ± 0·04
SD + 1% Peptone** (4)	0·58 ± 0·32[21·0]	0·03 ± 0·01	4·20 ± 1·22[10·5]	0·40 ± 0·02	9·53 ± 3·98[25·6]	0·37 ± 0·04	9·93 ± 3·92[28·2]	0·35 ± 0·03
SD + 1% Peptone** + 1 mM Trp† (4)	0·56 ± 0·29[15·9]	0·04 ± 0·02	4·94 ± 0·83[12·3]	0·40 ± 0·02	11·64 ± 1·41[28·1]	0·41 ± 0·03	13·7 ± 1·42[35·1]	0·39 ± 0·02
SD + 1% CAA†† (4)	0·46 ± 0·19[10·2]	0·05 ± 0·00	0·49 ± 0·21[1·0]	0·50 ± 0·03	0·66 ± 0·24[1·4]	0·48 ± 0·01	3·18 ± 14[7·7]	0·42 ± 0·02

*Mean ± SE: brackets [] indicate mean 3-MI values divided by A_{660nm} values.

†A filter-sterilized tryptophan solution was added to the cooled medium following autoclaving.

‡SD medium (pH 7·0) (per litre): 10 mmol glucose, 2 g yeast extract, 5 g ammonium sulfate and 2·69 g sodium bicarbonate, 1 ml trace mineral solution, 1·0 mg resazurin, 2·0 mg hemin, 40 ml salt solution and 1·0 g cysteine-HCl.

§Not detected.

¶SD medium prepared with 20 g l^{-1} yeast extract replacing 2 g l^{-1} yeast extract.

**SD medium prepared with 10 g l^{-1} peptone replacing glucose; tryptophan added where indicated.

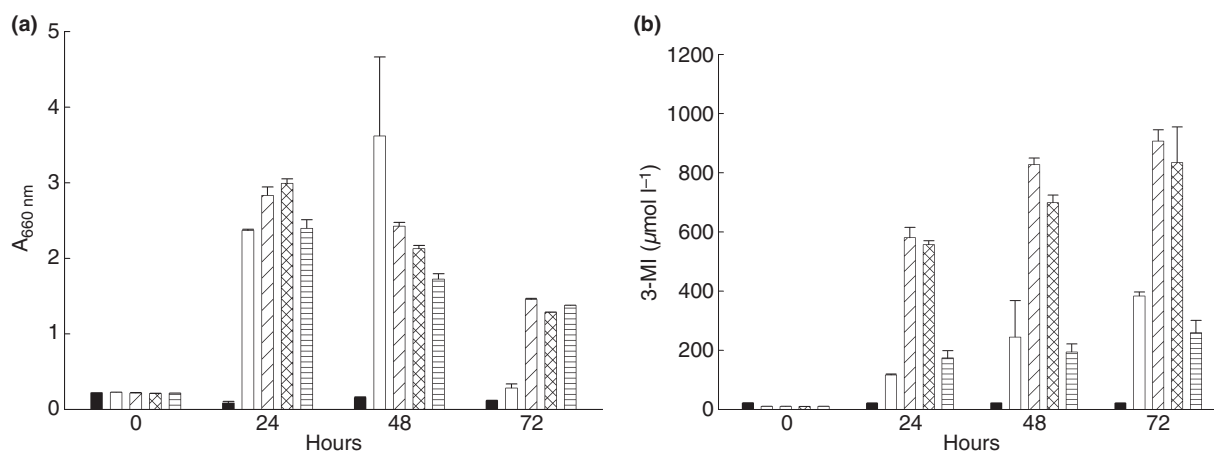††SD medium prepared with 10 g l^{-1} casamino acids replacing glucose.

Figure 1 Absorbance at 660 nm (a) and 3-methylindole levels (b) of *Cl. scatologenes* ATCC 25775 cultured in brain-heart infusion medium, pH 7.0, at various temperatures; 4°C, solid bars; 24°C, open bars; 33°C, angled-hatched bars; 37°C, cross-hatched bars, 40°C; horizontal-hatched bars. Data are mean ± standard error of three replicates..
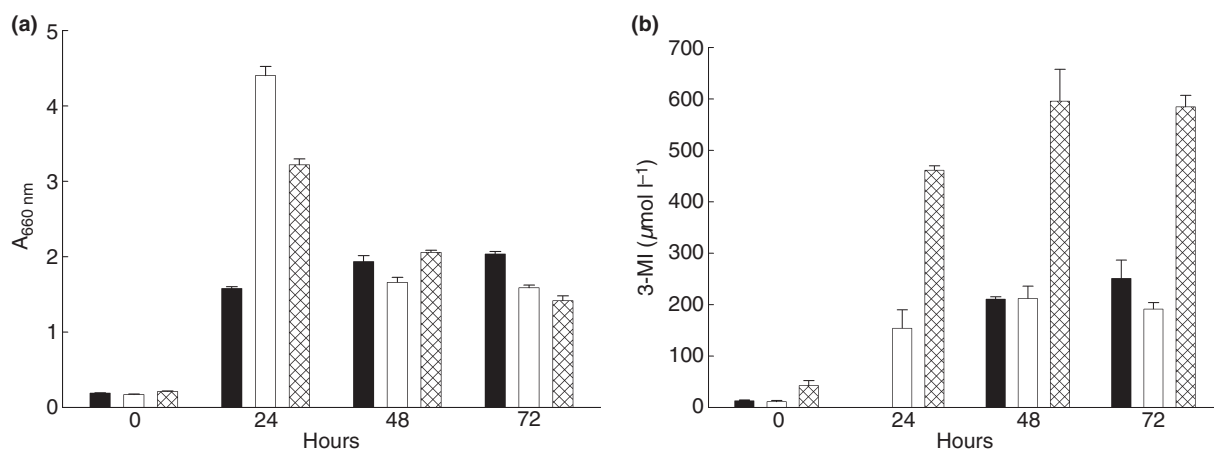

Figure 2 Absorbance at 660 nm (a) and 3-methylindole levels (b) of *Cl. scatologenes* ATCC 25775 cultured at 37°C in brain heart infusion medium with varying initial pH levels; pH 5.0, solid bars; pH 6.0, open bars; pH 7.0, hatched bars. Data are mean ± standard error of three replicates.

Table 2 Specific activity of 3-methylindole production by resting cells of *Clostridium scatologenes* ATCC 25775*

Growth medium	Nanomoles 3-MI h^{-1} OD A_{600nm}^{-1}†	
	+IAA	−IAA
BHI	0·035 ± 0·007‡,§	0·007 ± 0·002
TY	0·033 ± 0·012‡,**	0·008 ± 0·004
TY + 1 mM Trp	0·076 ± 0·031**	0·011 ± 0·005
SD	0·001 ± 0·001§	Tr.¶

*Cells were cultured in the growth medium for 24 h, washed three times and whole cells assayed at 37°C in the presence or absence of 1 mmol l^{-1} indole acetic acid, +IAA and −IAA, respectively.

†,**Nanomoles of 3-MI produced per hour per 1 ml of cells at 1·0 A_{660nm}.

‡Pairwise *t*-test not significant ($P \geq 0.05$).

§Pairwise *t*-test significant ($P < 0.05$).

¶Trace amounts detected.

and 0·076 ± 0·031 nmol 3-MI h^{-1} ml of culture at 1·0 $A_{660\,nm}^{-1}$, respectively. Similar to TY-grown cells, BHI-grown cells displayed a rate of 0·035 ± 0·007 nmol 3-MI h^{-1} ml of culture at 1·0 A_{660nm}^{-1}. SD-grown cells displayed a much lower rate of 0·001 ± 0·001 nmol 3-MI h^{-1} ml of culture at 1·0 A_{660nm}^{-1} (Table 2).

Discussion

Clostridium scatologenes ATCC 25775 was first recognized in 1925 for producing 3-MI and exhibiting a faecal odour (Fellers and Clough 1925) and recently has been shown to be a metabolically versatile organism. This strain grows using ethanol, formate, vanillate and produces hydrogen gas and is also an acetogen capable of reducing carbon dioxide to acetate (Kusel *et al.* 2000). Recent studies indicate *Cl. scatologenes* ATCC 25775, *Cl. drakei* SL1 and

Cl. carboxidivorans P7 form a unique, acetogenic clade within the clostridia (Liou *et al.* 2005). The 3-MI pathway is present in both *Cl. scatologenes* ATCC 25775 and *Cl. drakei* SL1 (Whitehead *et al.* 2008) in which tryptophan is deaminated to indole-3-pyruvic acid, which is decarboxylated to IAA and then decarboxylated to 3-MI (Whitehead *et al.* 2008). *Clostridium carboxidivorans* does not produce 3-MI (unpublished data). However, 3-MI is only partially responsible for the faecal odour of *Cl. scatologenes* ATCC 25775 as 4-methylphenol, which imparts a 'barnyard' odour (Mackie *et al.* 1998; Wright *et al.* 2005), propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid and caproic acid (Holdeman *et al.* 1977) are also produced.

Similar to earlier studies, *Cl. scatologenes* ATCC 25775 was cultured in rich or undefined medium. We have routinely observed 3-MI production and robust cell growth in BHI; thus, this medium was chosen for the experiments that varied pH and incubation temperatures. Data indicate *Cl. scatologenes* ATCC 25775 grows well at initial medium pH values of 5–7, consistent with previous reports. (Liou *et al.* 2005); however, we did not observe growth at an initial medium pH value of 8·0. *Clostridium scatologenes* ATCC 25775 produced 3-MI at all media pH values and temperatures that supported growth. A medium pH value of 7·0 proved optimal for 3-MI production while temperatures of 33°C or 37°C yielded optimal and similar levels of 3-MI. In BHI medium, final 3-MI concentrations varied considerably among experiments. For example, Fig. 1b shows when this strain is cultured for 48 h at 37°C at pH 7·0, 3-MI is 698 μmol l^{-1}, whereas in Table 1, those conditions yield levels of 307 μmol l^{-1} 3-MI. For comparative purposes and to attempt to reduce the intra-experimental variation of 3-MI production, we cultured *Cl. scatologenes* ATCC 25775 in TY medium. In this medium, maximum 3-MI levels decreased when compared with BHI but still displayed substantial variation. For example in Table 1, culturing in TY medium produced 104·86 μmol l^{-1} 3-MI but with the large SE of 55·57. One complicating factor when using rich medium, such as BHI or TY, is the amount of tryptophan available to the cells is unknown. BHI and TY measure 294 ± 12 and 340 ± 10 μmol l^{-1} free tryptophan, respectively, however, this measurement does not consider the tryptophan available from peptides. The tryptophan available in peptides is difficult to control and could contribute to the observed variation. Alternatively, the variation observed in rich medium could be indicative of true metabolic variability because the reproducibility of 3-MI measurements improved when more austere media were used and maximum 3-MI levels decreased. For example, SD medium produced 0·08 μmol l^{-1} 3-MI at 48 h but displayed a SE of only 0·01 (Table 1). These data suggest maximum levels of 3-MI produced by the cells vary among media and is most pronounced when 3-MI is produced at high levels but is also coincident with higher cell densities.

When 3-MI concentrations are standardized against cell densities (Table 1), cells cultured in rich medium produce more 3-MI per cell. For example, cells cultured for 72 h in BHI exhibited 3-MI : cell density ratios 332-fold higher than cells grown using SD medium (199·5 *vs* 0·6). This effect could be due to the presence of more tryptophan available in rich medium as opposed to austere medium or the enzymes responsible for 3-MI production are expressed to a greater extent in BHI-grown cells than SD-grown cells. However, measurements taken from 48 h of growth indicate that 3-MI : cell density ratios of TY and TY + 1 mmol l^{-1} tryptophan were similar (133.2 *vs* 135.5), suggesting the presence of exogenous tryptophan did not influence 3-MI production. Thus, it is difficult to draw a conclusion based on these data.

To address this problem, whole cell assays measuring specific activity of IAA decarboxylase were performed (Table 2) and the results support the notion that enzymes responsible for 3-MI production were up-regulated in rich medium. Rates of 3-MI production in BHI-, TY- and TY + 1 mmol l^{-1} tryptophan-grown cells were 35-, 33- and 76-fold higher than in SD-grown cells, indicating IAA decarboxylase is up-regulated in rich medium. These data also show that the role of tryptophan in the up-regulation is unclear. Specific activity values in TY plus 1 mmol l^{-1} tryptophan were 2·3-fold higher in TY-grown cells, but this increase was not statistically significant. Furthermore, these data suggest the variation of maximum 3-MI levels observed in Fig. 1 and Table 1 was likely due to differing degrees of expression of the 3-MI production pathway as well as varying levels of available tryptophan.

To assist in resolving the role of tryptophan in 3-MI expression, the laboratory is currently developing a defined medium for *Cl. scatologenes* ATCC 25775 in which the concentration and presence of nutrients can be controlled. In addition, these data suggest 3-MI production can be more precisely controlled in an applied setting, such as a hog waste lagoon. 3-MI production is inhibited by acidic conditions, is not a required metabolic function and can be down-regulated. For example, if those metabolic factors which down-regulate 3-MI production are elucidated, feed or lagoon additives may be developed which 'turn-off' this pathway thereby diminishing 3-MI levels and emissions.

Acknowledgements

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